Role of intestinal brush border membrane aminopeptidase N in dipeptide transport

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The kinetics of uptake of radioactive label from [U-^{14}C]Gly, L-[4,5-^{3}H]Leu and the dipeptide [^{14}C]Gly–L-[4,5-^{3}H]Leu by the brush border membrane vesicles of porcine small intestine have been studied. The effect of aminopeptidase N inhibitors and leucine-binding protein on accumulation rates has also been tested. Comparison of the kinetic parameters for uptake and hydrolysis of Gly–L-Leu makes it possible to conclude that the dipeptide transfer includes two conjugated steps, viz., hydrolysis catalysed by aminopeptidase N and transport of the resultant free amino acids by a specific carrier.

Brush border membrane *Aminopeptidase N* *Dipeptide* *Amino acid transport*

1. INTRODUCTION

Aminopeptidase N of enterocytes plays an important role in food protein degradation at its final stages [1]. It is suggested that in addition to hydrolysis of short oligopeptides, this enzyme participates in peptide transport across the membrane of microvilli [1–3]. At present 4 possible mechanisms of dipeptide transport are discussed (fig.1) [4].

(I, II) Transfer by a special peptide carrier with the following hydrolysis up to amino acids with cytosol peptidase or by the hydrolytic site of the carrier itself, respectively.
(III) Peptide hydrolysis catalysed by aminopeptidase N, and the independent transport of the amino acids formed by the amino acid carrier in an Na^+-dependent process.
(IV) Peptide hydrolysis catalysed by aminopeptidase N with transferal of the amino acids to the specific carriers inside the membrane. In this case aminopeptidase N itself can apparently serve as such a carrier [5–7].

Despite numerous investigations on amino acid and peptide transport through the intestinal brush border membrane [4,6–12], no choice has been made till now among the 4 possible mechanisms of dipeptide transport.

As an approach to this problem we compare the kinetics of dipeptide hydrolysis by the brush

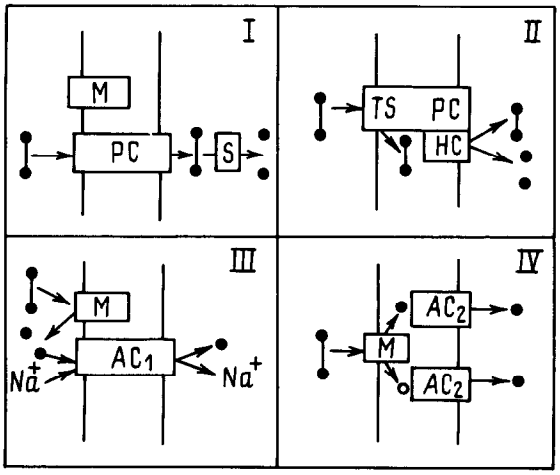

Fig.1. Peptide transport mechanisms. M, membrane aminopeptidase; S, cytosol peptidase; AC_1, Na^+-dependent amino acids carrier; AC_2, specific amino acids carrier; PC, peptide carrier; TS, transferring site; HS, hydrolytic site; (●—●) peptide; (●) amino acid.

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border vesicular aminopeptidase N of porcine small intestine with kinetics of uptake both of free amino acids and of the dipeptide by vesicles. The influence of some substances which interact with the aminopeptidase and with the substrate on the transport rate are also studied.

Our results give evidence in favour of mechanism IV which includes dipeptide hydrolysis by aminopeptidase N and subsequent transfer of the resultant free amino acids to specific carriers.

2. MATERIALS AND METHODS

Brush border membrane vesicles of porcine small intestine were obtained from mucosa as in [13]. The labeled amino acids, L-[4,5-^{3}H]leucine (170 Ci/mmol) and [U-^{14}C]glycine (107 mCi/mmol) were purchased from Amersham. The [U-^{14}C]Gly–L-[4,5-^{3}H]Leu dipeptide (125 μCi/mmol of ^{14}C and 221 μCi/mmol of ^{3}H) was obtained from the corresponding protected amino acids by the dicyclohexylcarbodiimide method. Unlabeled Gly, L-Leu and Gly–L-Leu were from Sigma, *o*-phenanthroline from Chemapol and puromycin from Reanal. L-Leucine *p*-nitroanilide was synthesized as in [14]. Leucine-isoleucine-valine binding protein (LIV-protein) from *E. coli* was isolated as in [15,16] by S.D. Trakhanov.

Aminopeptidase N activity in the vesicles was assayed spectrophotometrically on L-leucine *p*-nitroanilide as substrate (at 405 nm) with a Gilford 2400-2 unit [17]. Protein concentration in the vesicles was measured by means of Coomassie brilliant blue G-250 [18]. The Gly-Leu dipeptide (1×10^{-4}–1×10^{-2} M) was hydrolysed in 10 mM Hepes–Tris buffer containing 100 mM mannitol, 100 mM NaCl, 0.1 mM $MgSO_4$ (pH 7.5, buffer A), using newly prepared vesicles (protein concentration, 0.55 mg/ml). To stop the reaction NH_4OH was added up to pH 10.5–11 and the reaction mixture was boiled for 2–3 min. The unhydrolysed dipeptide concentration was determined by HPLC on an Altex model 334 unit with an Ultrasphere ODS column (5 μm, 4.6 × 250 mm) in methanol–0.01 M phosphate buffer (pH 2.6, 4:6).

To measure the time course of uptake of radioactivity from free amino acids and dipeptide inside brush border membrane vesicles, a suspension of fresh vesicles (0.3 ml) was added to 0.7 ml solution of radioactive amino acids or dipeptide in buffer A at 27°C, so that the substrate and protein concentrations in the incubation medium were 10^{-2}–5×10^{-4} M and 1.6 mg/ml, respectively. The incubation mixture was diluted with a 25-fold volume of ice-cold buffer [10 mM Hepes–Tris (pH 9.2), 100 mM mannitol, 200 mM NaCl, 100 mM $MgSO_4$ (buffer B)] and filtered immediately through GF/F filters (Whatman, ϕ 2.4 cm), then rinsed with 100 ml ice-cold buffer B. The dried filters were placed in counting vials and 5 ml scintillation fluid (Biosolve-toluene) were added. The radioactivity was counted with an Intertechnique SL-30 counter. Correction for non-specific binding of amino acids and the peptide was made using a similar test, replacing native vesicles by those having lost their transport activity, but retaining the hydrolytic properties. Such vesicles were obtained by keeping for 24 h at 20°C. The divergence of the two parallel tests did not exceed 8%. The experiments were repeated 4–6 times. To detect the unchanged dipeptide inside the vesicles, the same procedure was repeated with unlabelled Gly-Leu dipeptide. The vesicles collected on the GF/F filters were washed with hot 30% methanol (5 ml). The eluate was boiled for 3 min. The combined eluate of 5 tests was lyophilised. The dipeptide and amino acid content in the dry residue was determined by chromatography on a Biotronic amino acid analyser and by HPLC. Here unspecific amino acid retention by the filter was taken into account. To study the effect of LIV-protein on the rate of dipeptide uptake, the vesicle suspension (0.3 ml) was added to a solution of [^{14}C]Gly–[^{3}H]Leu dipeptide and lyophilised LIV-protein in buffer A (0.7 ml), the resultant concentrations being 1.6 mg/ml for the vesicular protein, 1×10^{-3} M for the dipeptide and 2×10^{-5} M for the LIV-protein. The solution obtained was incubated for 10 min at 27°C.

3. RESULTS AND DISCUSSION

When brush border membrane vesicles were incubated with the Gly-Leu dipeptide two processes were observed: hydrolysis of the dipeptide up to free amino acids and uptake of the amino acids formed (and possibly of the intact dipeptide) by vesicles.

To choose between the above-mentioned transport mechanisms one should know whether aminopeptidase N participates in transferring the radioactive labels of both free amino acids and the dipeptide. For this purpose we studied the effect of the enzyme inhibitors, puromycin and *o*-phenanthroline, on the uptake of free amino acids and of the [^{14}C]Gly–[^{3}H]Leu peptide by vesicles. Fig.2a,b shows that these inhibitors do not affect radiolabel accumulation of the free amino acids, but considerably reduce radiolabel from the dipeptide. Thus, aminopeptidase N participates somehow in the transport if the dipeptide is taken as a substrate.

Moreover, according to fig.2b, glycine and L-leucine from the dipeptide are accumulated in vesicles at different rates. It seems reasonable to compare the kinetic parameters measured under identical conditions for both hydrolysis and uptake of the dipeptide with the parameters for transfer of free amino acids. The experiments were performed at constant NaCl concentration. The rates of both hydrolysis and uptake did not change during 40–90 s incubation. The kinetic curves were treated as in [19]; the kinetic parameters were computed by the least-squares technique.

Fig.3 presents typical plots of v *vs* $v/[S]_0$ and table 1 summarizes the parameters which characterize the uptake and hydrolysis processes. The maximum rate of dipeptide hydrolysis is, by a factor of 10^3, greater than the radiolabel accumulation rate from the dipeptide in vesicles whereas the Michaelis constants for hydrolysis and uptake are comparable. The accumulation of glycine and leucine from the dipeptide proceeds at varying maximum rates. The Michaelis constants and maximum rates for the transport of free amino acids are higher than those of the dipeptide amino acids. All these data are inconsistent with mechanisms I and II because, on the one hand, they stipulate the same rate for accumulation of different dipeptide amino acids and, on the other, they do not include aminopeptidase N as a participant in the total transfer process. Finally, the intact dipeptide was not detected inside vesicles by amino acid analysis (fig.4) and HPLC, in contradiction with mechanisms I and II. Vesicles contain no peptidase inside. Destruction of vesicles by Triton X-100 (1% solution) did not lead to an increase of the rate of leucine *p*-nitroanilide

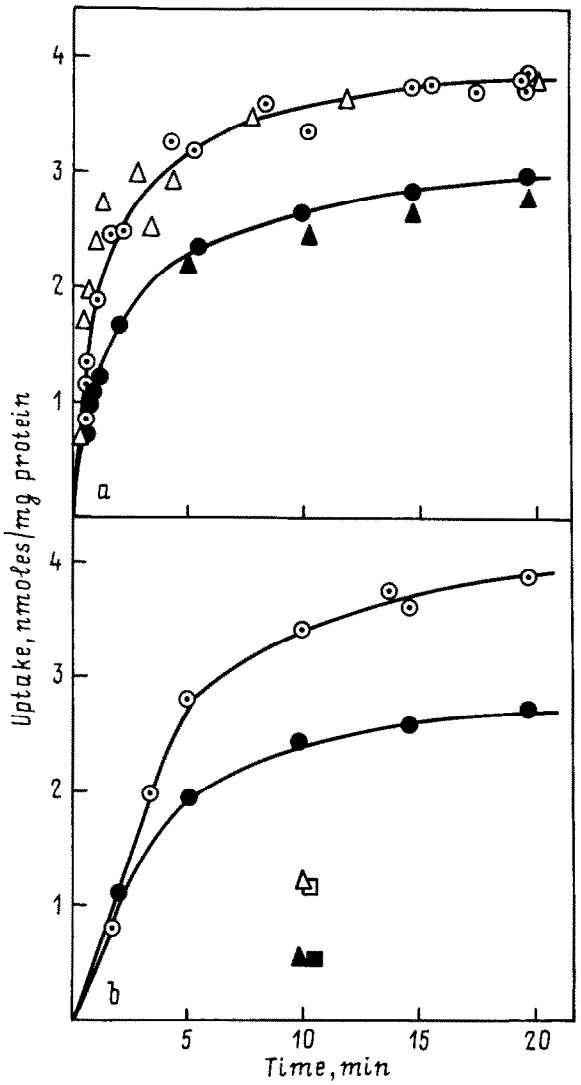

Fig.2. Accumulation of radioactive label during incubation of vesicles with amino acids: (a) [^{14}C]Gly (●), [^{3}H]Leu (⊙); (b) the [^{14}C]Gly–L-[^{3}H]Leu dipeptide without (●,⊙) and with hydrolysis inhibitors: puromycin (▲, △) and *o*-phenanthroline (■, □). Concentrations of amino acids and the dipeptide,2.5 mM; concentration of vesicle protein, 1.6 mg/ml.

hydrolysis, this fact being in accordance with data of [8], giving an additional argument against mechanism I.

The kinetic data are also in disagreement with mechanism III. Despite the considerably higher maximum rate of dipeptide hydrolysis compared to the rate of label accumulation from the dipep-

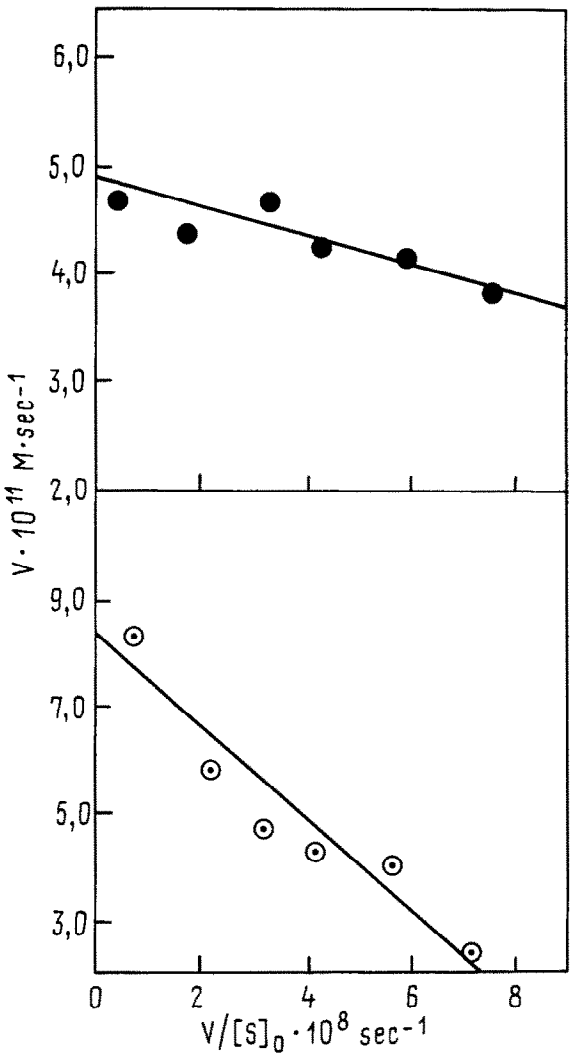

Fig.3. Eadie-Hofstee plots for accumulation of ^{14}C (●) and ^{3}H (⊙) in vesicles from [^{14}C]Gly-[^{3}H]Leu dipeptide.

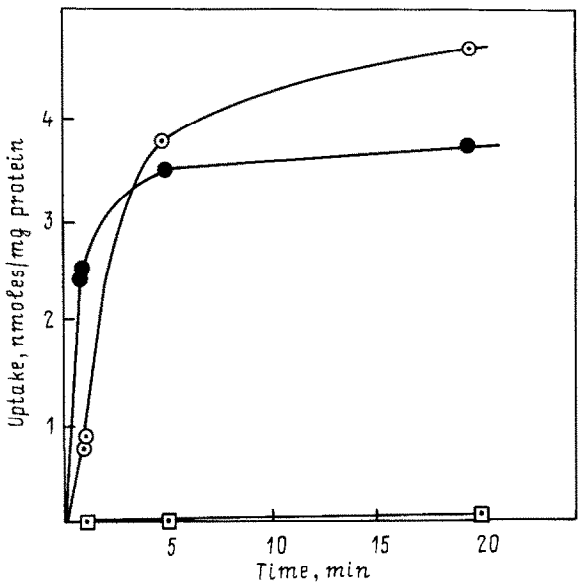

Fig.4. Accumulation of Gly (●), Leu (⊙) and Gly-Leu (□) by vesicles after their incubation with Gly-Leu dipeptide (data of amino acid assay). Concentration of Gly-Leu, 5 mM and of vesicle protein, 2.5 mg/ml.

tide, it is still insufficient to provide the observed initial rate of Gly and Leu uptake by vesicles since the concentration of free amino acids formed is too low. In fig.5 the experimentally observed initial rates of amino acid uptake from the dipeptide are compared with those calculated according to mechanism III from the rate of dipeptide hydrolysis. The theoretical values are lower than the experimental ones by a factor of 10^5–10^6.

Mechanism III involves formation of free amino acids in solution due to dipeptide hydrolysis and

Table 1

Kinetic parameters for hydrolysis of Gly-Leu by aminopeptidase N of brush border membrane vesicles and for uptake of radioactive label by vesicles from the [^{14}C]Gly-[^{3}H]Leu dipeptide and from free [^{14}C]Gly and [^{3}H]Leu

Substrate	Process	V_{max} ($\times 10^9$) (M/min per mg protein)	K_m ($\times 10^3$) (M)
Gly–L-Leu	Hydrolysis	2500 ± 500	0.5 ± 0.1
[^{14}C]Gly–L-[^{3}H]Leu	Accumulation of ^{14}C	2.9 ± 0.5	0.14 ± 0.03
	Accumulation of ^{3}H	5.1 ± 0.6	0.9 ± 0.05
[^{14}C]Gly	Accumulation of ^{14}C	6.6 ± 1	5.6 ± 0.5
[^{3}H]Leu	Accumulation of ^{3}H	55.8 ± 5	12.4 ± 1

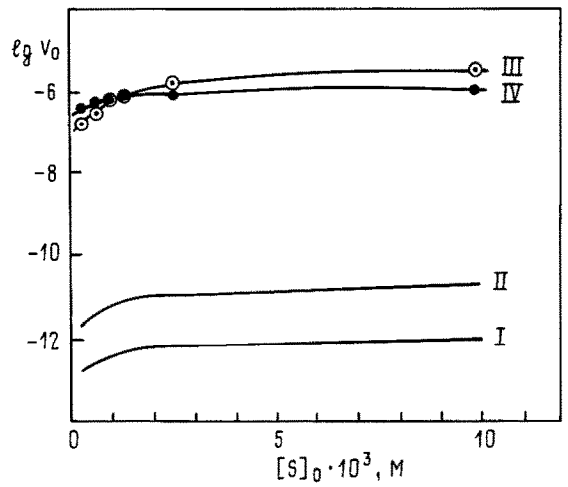

Fig.5. Dependence of the initial rate of dipeptide uptake on its concentrations (I: [^{14}C]Gly, II: [^{3}H]Leu), calculated according to mechanism III and corresponding experimental data (III: [^{14}C]Gly, IV: [^{3}H]Leu). Theoretical curves were calculated from the data on dipeptide hydrolysis rate and uptake rates of free amino acids (for 1 min incubation).

their subsequent transport across the membrane. Therefore, if a substance binding amino acids (but not the dipeptide) is introduced into the system, the rate of label accumulation from the dipeptide should decrease. Such a binding substance for leucine may be, for instance, LIV-protein from *E. coli*, the K_d for the leucine–LIV-protein complex being about 1×10^{-7} M [16]. We found that the introduction of LIV-protein into the Gly-Leu dipeptide–membrane vesicle system does not decrease leucine accumulation as compared with that of glycine from the dipeptide (table 2). It should be noted that the calculated rate of amino acid diffusion into the bulk of the solution is, by a factor of 10^8, higher than the rate of label uptake inside vesicles. The LIV-protein concentration (2×10^{-5} M) was low enough for the dipeptide concentration to decrease slightly due to their non-specific interaction ($K_d \sim 1 \times 10^{-3}$ M), but all leucine in the solution produced due to hydrolysis was bound in a complex with the LIV-protein. In this case dipeptide glycine could serve as an internal control.

The data obtained comply with mechanism IV which includes dipeptide hydrolysis by aminopeptidase N inside the membrane and the subsequent transfer of resultant amino acids by a specific carrier. We cannot exclude at the moment that transmembrane aminopeptidase N itself may serve as such a carrier (cf. [5–7]). The rate-determining step of the whole dipeptide transport is the translocation process ($V^{hyd}_{max}/V^{tran}_{max} = 10^3$). So, the main fraction of amino acid formed due to hydrolysis of the dipeptide is accumulated in the bulk of the solution. At the initial stage of the process their concentration is low and does not influence the dipeptide uptake. At high concentrations free amino acids may be transferred across the membrane by the same carrier system. The problem of the different sensitivity of the peptide and amino acid transport to an Na^+ concentration gradient [4] still calls for further investigation.

Table 2

Accumulation of radioactive label by vesicles during their incubation with [^{14}C]Gly–L-[^{3}H]Leu dipeptide in the presence of LIV-protein

Conditions	Accumulation (nmol/mg protein)		% of uptake	
	[^{3}H]Leu	[^{14}C]Gly	[^{3}H]Leu	[^{14}C]Gly
Without LIV-protein	1.92	3.90	100	100
With LIV-protein	1.63	3.28	84	83

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